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(54) Title: INACTIVATION OF CYTOTOXIC DRUGS

(57) Abstract

A three component kit of parts for use in a method of destroying target cells in a host is provided. The first component comprises a target cell-specific portion and an enzymatically active portion capable of converting a cytotoxic pro-drug into a cytotoxic drug. The second component is a cytotoxic pro-drug convertible by said enzymatically active portion to the cytotoxic drug. The third component comprises a portion capable of at least partly restraining the component from leaving the vascular compartment of a host when said compound is administered to the vascular compartment, and an inactivating portion capable of converting the cytotoxic drug into a less toxic substance.

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INACTIVATION OF CYTOTOXIC DRUGS

This invention relates to cytotoxic drug therapy and, more specifically, to the inactivation of cytotoxic drugs to limit their undesirable side effects.

One of the main limitations of conventional cytotoxic therapeutic agents is their lack of discrimination between cancer cells and normal replicating cells which are essential for normal tissue integrity and body function. The effects of cytotoxic agents on these normal tissues limits the dosage of cytotoxic therapy or the duration of its administration. Cytotoxic drug therapy is therefore interrupted at frequent intervals to allow normal tissue recovery. Such interruption also allows recovery of surviving cancer cells and may be important in allowing the emergence of drug resistance.

The duration of drug free intervals in therapy usually exceeds the duration of cytotoxic agent administration. This applies to all forms of cytotoxic therapy but is particularly evident in the case of those agents that interfere with DNA replication during S phase of the cell cycle.

A method for the treatment of various cancers known as Antibody-Directed Enzyme Pro-drug Therapy (ADEPT) has been described (Bagshawe 1987 *Br. J. Cancer* **56**, 531-2; 1989 **60**, 275-281) and is undergoing early clinical trial in which an antibody, or antibody fragment, directed at a tumour-associated antigen expressed by at least some cells in the cancer target, is conjugated to an enzyme and used to convey that enzyme to the cancer sites. The enzyme in the conjugate is matched by a subsequently administered prodrug which is a relatively non-toxic substance and is a substrate for the enzyme, the end-product of the reaction being an active cytotoxic drug which is able to diffuse through the tumour and reach cells that do not express the target antigen. In such therapy it is desirable that the enzyme used should not normally be present

in significant amounts in human body fluids and that the prodrug is only subject to conversion to active drug by the targeted enzyme (WO88/07378).

In the example of choriocarcinoma xenografts in nude mice, cited in WO 88/07378, clearance of the antibody enzyme conjugate is accelerated by the presence of relatively large amounts of the target antigen in the plasma which results in immunoconjugate formation. In this case, the accelerated clearance did not prevent localisation of the antibody enzyme conjugate at tumour sites although, in general, rapid clearance of the antibody enzyme conjugate from plasma results in poor tumour localisation.

In another exemplification of the antibody enzyme pro-drug principle (Senter *et al* (1988) *Proc. Natl. Acad. Sci. USA* **85**, 4842-4846 the targeted enzyme, alkaline phosphatase, is widely distributed in body tissues including plasma so that pro-drug activation must occur at all these sites in addition to that generated at tumour sites. The tumour generated increment of active drug under such conditions can only be small.

The objective of an antibody-enzyme pro-drug approach is to confine the action of cytotoxic agents to tumour sites. This would be advantageous in allowing a greater concentration of drug to act at tumour sites without incurring greater toxic effects on normal tissues. Also, many cytotoxic drugs currently in use are carcinogenic and survivors from one cancer may succumb to a cancer induced by treatment of the former cancer through the effect of the drugs or radiation on normal tissues; restricting mutagenic drugs to cancer sites would reduce this risk of iatrogenic cancer.

A method of treatment in which an active cytotoxic drug is generated from a less toxic pro-drug by catalytic action can only achieve the objective, as defined, if the catalyst is confined to the target site(s). When an antibody or

fragment thereof, or a conjugate comprising an antibody or fragment thereof and an enzyme, is injected into a host bearing a tumour which expresses on the cell surface an antigen corresponding to the antibody, the antibody or conjugate preferentially localises at tumour sites. That is to say, the concentration of enzyme at tumour sites is likely to be higher than in other tissues after an interval of hours or days. However, most of the administered antibody or conjugate is retained in other tissues, including the blood to some extent. Although a patient with terminal cancer may have several kilograms of tumour it rarely represents more than 10% of body weight and treatment is generally undertaken when the body burden of cancer ranges from a few grams to about 1 kilogram. Thus the advantage of a higher concentration of the enzyme achieved at tumour sites by the antibody-enzyme conjugate is offset by the much greater volume of normal tissues retaining a low concentration of the enzyme. The enzyme-pro-drug reaction takes place in the interstitial fluid (extracellular space) which in a tumour of 1 kilogram is unlikely to exceed 1-200 millilitres whereas the plasma volume of an average adult male is 2-3 litres and the total extracellular space about 15 litres. A consequence of these considerations is that a method of treatment based on an antibody-enzyme conjugate, and a subsequently administered pro-drug, is still subjected to a dose limiting effect through cytotoxic drug action on normal tissues. It has been shown in nude mice bearing human colon cancer xenografts that if a pro-drug is given when enzyme is still present in plasma in significant amount that the pro-drug is rapidly activated with fatal effects (Bagshawe (1989) *Brit. J. Cancer.* **60**, 275-281).

Since antibody-enzyme conjugates generally reach their maximum concentration at tumour sites within 12-24 hours and since they may take several days to clear from plasma and other body fluids, it has also been shown that it is advantageous to accelerate the clearance of antibody-enzyme conjugate and to inactivate the specific enzyme present in the blood. Several means by which

this may be achieved have been described (WO89/10140).

Clearing and/or inactivating the enzyme in plasma has a marked effect, allowing a greater amount of pro-drug to be administered. Considerable clinical and experimental experience has been gained with the use of a galactosylated anti-enzyme antibody which rapidly inactivates enzyme in blood after tumour localisation of the enzyme has been achieved. The galactosylation of the anti-enzyme antibody results in its rapid clearance from the blood through take-up by galactose receptors on hepatocytes and is necessary to prevent its escape from the vascular compartment onto the tumour where it would inactive the enzyme. The fall in plasma enzyme concentration with the use of such a method. in the other methods described in WO 89/10140 causes antibody enzyme conjugates not bound to antigen in normal tissues to diffuse back into the plasma compartment. The enzyme returning to blood can, for instance, be inactivated by a slow infusion of anti-enzyme antibody. Although these accelerated clearance and inactivating methods make antibody enzyme pro-drug therapy widely applicable. some active drug still enters the vascular compartment and reaches cell renewal tissues. particularly the haemopoietic tissues. and can be dose limiting. Where a tumour is relatively large a significant amount of active drug will enter the blood by direct diffusion. or via the lymphatic chain. in addition to any active drug formed by residual enzyme activity in normal tissues.

Using these methods the generation of a cytotoxic agent can be restricted to those sites where the specific enzyme is located and predominantly to cancer sites.

A cytotoxic drug generated anywhere in the body diffuses through the extracellular fluid and is able to reach nearby cells which do not express the marker antigen to which the antibody is directed. It may also pass back into

the vascular compartment and then be conveyed to cell renewal tissues. If the tumour mass is large and if the drug has a half-life of more than a few seconds then effects on normal tissues may still prove dose-limiting.

It is therefore undesirable for active drug to be present in the blood.

Anti-tumour antibodies exist which show little or no binding to haemopoietic tissue, but since haemopoiesis occurs within capillaries, or in proximity to fenestrated capillaries, haemopoietic cells are highly vulnerable to the action of cytotoxic agents present in blood.

This blood-mediated effect can be limited by ensuring that the active drug has a short half-life (Bagshawe 1987). A drug generated at any point in the body can reach, via the circulating blood, any other tissue within 20-30 seconds. Some valuable cytotoxic agents possess relatively long half-lives and in the absence of an additional mechanism they may be excluded from consideration. Also, it may prove difficult to identify highly active short half-life drugs that can be generated from non-toxic prodrugs. A further consideration is that a very short half-life may limit the concentration of active drug that can be achieved at sites within a tumour mass that are remote from sites of antigen expression and therefore of active drug generation.

The objective of the present invention is to inhibit, at least partially, in the blood any active drug which is present in blood, whether it has been generated in blood, or gains access to the blood, from the tumour or normal tissue.

One aspect of the present invention provides a compound comprising a portion capable of at least partly restraining the compound from leaving the vascular compartment of a host when said compound is administered to the vascular compartment and an inactivating portion capable of converting a cytotoxic agent

into a less toxic substance.

The method used to retain an inactivating agent in the vascular compartment will be determined in part by the nature of the inactivating agent which in turn may be determined by the nature of the active drug.

The retaining component must be biologically compatible with all the functions of blood and the body's organs and should be biodegradable although degradation may take place slowly. It is a requirement that entry of the retaining component into tumour extracellular should be minimal. It follows from the latter requirement that the inactivating portion should not be released in a free state from the retaining portion unless the free inactivating portion is rapidly removed from the blood thus limiting its access to tumour extracellular space.

The restraining portion may be a red blood corpuscle. Suitably, the compound may be contained on or within red blood corpuscles by utilising techniques known in the art for causing molecules to enter into red blood corpuscles. The inactivating portion may be attached to the erythrocyte membrane or may be made to enter the erythrocyte and be retained therein.

A method of introducing desired agents into mammalian red blood cells without unacceptable loss of cell contents has been described (US 4931276 incorporated herein by reference). This method comprises (a) suspending and incubating the cells in a solution containing a compound which readily diffuses into and out of the cells, the concentration of the compound being sufficient to cause diffusion into the cells so that the contents of the cells become hypertonic; (b) rapidly creating a trans-membrane osmotic gradient by diluting the solution containing the hypertonic cells with an isotonic aqueous medium in the presence of at least one agent to be introduced to cause diffusion of water into the cells

with consequent swelling and increase in permeability of the outer membranes; and (c) maintaining the increase in permeability of the membranes for a time sufficient only to permit transport of the agent into the cells and diffusion of said compound out of the cells. This method is especially useful with the "osmotic pulse" mechanism of US4478824 for loading the cells.

US4652449 (incorporated herein by reference) describes a further method for encapsulation of a biologically-active substance in mammalian erythrocytes which comprises (a) continuously feeding an aqueous suspension of erythrocytes into the main compartment of a dialysis unit, the second compartment of which contains an aqueous solution which is hypotonic with respect to the erythrocyte suspension so as to cause lysis of the erythrocytes and (b) causing the erythrocyte lysate to be in contact or contacted with the biologically active compound(s) and then resealing the erythrocyte membranes by increasing the oncotic and/or osmotic pressure of the lysate.

Alternatively, the restraining portion may be a liposome, or a high molecular weight (>25,000 Daltons, preferably at least 40.000 Daltons) polymer such as dextran, or a protein such as macroglobulin.

Liposomes with prolonged circulation times are also potential carriers for the inactivating portion. Liposomes with prolonged circulation times have been constructed in various ways. These include the use of ganglioside GM1, or a mixture of sphingomyelin, egg phosphatidylcholine and cholesterol, or other relatively rigid carrier lipids. For each type of liposome, optimisation by sizing is also desirable to optimise prolonged intravascular residence. Gabizon and Papahadjopoulos (1988) *Proc. Natl. Acad. Sci. USA* **85**, 6949-6953) found a mean particle diameter of 100 nm to be optimal. However, liposomes with a prolonged circulation time are suitable only if they show no significant uptake in tumours.

Dextrans are polysaccharides consisting of α-D-glucose units joined predominantly by 1-6 linkages. Partially hydrolysed and fractionated dextran has been widely used as a blood plasma expander. They are cleared by dextranases which are present in the liver, spleen, kidney, intestinal mucosa and colon (Rosenfeld *et al* (1959) Biokhimia [Engl] **24**, 965-970; Ammon (1963) *Enzymologia* **25**, 245-251; Serry & Hehre (1956) *J. Bacteriol.* **71**, 373-380). Dextrans are widely available with mean molecular weights of 40 kD (Gentran 40. Rheomacrodex), 70-75 kD (Gentran 70 Macrodex) and 110 kD. They are potentially antigenic and some people have pre-existing antibodies, but reactions to dextrans are reported to be no higher than to other widely used pharmaceutical agents and are mild in character (Goodman and Gilman. *The Pharmacological Basis of Therapeutics*. 8th Edition. Pergamon Press. New York, Oxford, 1990).

Other polymeric drug carriers have been developed from carbohydrates, peptides and lipids. Some of these may be suitable alternative restraining portions for the inactivating portion provided they fulfil the criteria defined here.

The restraining portion may advantageously have a negative charge and may have low lipophilicity.

Suitably, the restraining portion is biodegradable.

By "biodegradable" we mean that the retaining portion is degraded in the body of the patient, and therefore has a relatively short half-life. for example less than 72 hours, preferably less than 48 hours.

A second aspect of the present invention provides a method of inactivating a cytotoxic agent in the vascular compartment of a host comprising administering

to the host said compound.

Thus, it is preferred that the active drug is an alkylating agent and the method of intravascular inactivation is by reaction with reduced glutathione, a reaction which is catalysed by glutathione-S-transferase. The non-protein thiol glutathione is regarded as the main endogenous reducing agent. Both glutathione and glutathione-S-transferase are present in normal erythrocytes, and it has been shown that glutathione levels in erythrocytes correlate with responses to conventional chemotherapeutic agents in patients with breast cancer, high levels being associated with poor response (Herbergs *et al* (1992) *The Lancet* 339. 1074-6). It has also been shown that the glutathione content of erythrocytes can be substantially increased (3- to 4-fold) by a procedure of hypertonic dialysis and isotonic resealing (Fazi *et al* (1991) *Biotech and App. Biochemistry* 14. 60-68).

Inactivation of alkylating agents in the blood is therefore possible using erythrocytes overloaded with glutathione. For this to be effective, the active drug is required to be more readily inactivated than the pro-drug from which it is derived.

It is further preferred that antibodies which discriminate between an active drug and its pro-drug are administered. Since a pro-drug, usually, possesses a cleavable moiety which is not present in the active drug, this provides a structural difference between the pro-drug and drug such that the said antibodies can bind only to the drug.

Monoclonal antibodies have the advantage that they can be humanized thereby limiting their immunogenicity. Such an antibody needs to be retained within the vascular compartment since it would otherwise inactivate active drug at tumour sites. Restriction to the vascular compartment may be achieved by

galactosylating the antibody which confers a short half-life through uptake by hepatocyte galactose receptors (Sharma *et al* (1990) *Brit. J. Cancer* **61**, 659-662). The more galactose moieties on the antibody the shorter the half-life. The short plasma half-life limits tissue penetration but to be effective would require continuous intravenous infusion throughout the period of active drug generation.

Alternatively, antibody may be conjugated to a slowly biodegradable polysaccharide such as a dextran.

It is still further preferred that an enzyme which degrades the active drug, or renders it unable to cross cell membranes, and which has no effect, or much less effect, on the pro-drug is administered. In certain circumstances it is advantageous to use an inactivating enzyme compared to an inactivating antibody since one enzyme molecule may inactivate a large number of active drug molecules whereas an antibody would act stoichiometrically (ie one antibody is required to inactivate one active drug molecule). A non-human enzyme has the disadvantage of being immunogenic and therefore requires some form of immunological control but human enzymes including glucuronidases, kinases, sulphatases and other drug-inactivating enzymes may be used depending on the active drug substrate. Humanized catalytic antibodies would be advantageous.

An enzyme may be incorporated into erythrocytes by the osmotic technique described herein for the incorporation of glutathione, or incorporated in liposomes with prolonged circulation time. Alternatively, an enzyme may be retained within the vascular compartment by conjugation to one of the many types of slowly biodegradable polymeric drug carrier (Krinick & Kopeček (1991) in *Targeted drug delivery*, pp 105-179 (Ed. R.L. Juliano), Springer Verlag New York).

A third aspect of the present invention provides a three component kit of parts comprising a first component comprising a target cell-specific portion and an enzymatically active portion capable of converting a pro-drug into a cytotoxic drug; a second component that is a pro-drug convertible by said enzymatically active portion to the cytotoxic drug; and a third component comprising a portion capable of at least partly restraining the component from leaving the vascular compartment of a host when said compound is administered to the vascular compartment, and an inactivating portion capable of converting the cytotoxic drug into a less toxic substance at non-target cell sites.

In this way, the action of the cytotoxic agent may be sustained at tumour sites for significantly longer periods than is possible by conventional therapy, such that the clinical usefulness of the drug is substantially increased. In accordance with the method of the present invention, a cytotoxic agent is generated at target cell sites, whereas any drug which reaches the vascular compartment is at least partially destroyed or inactivated, thereby allowing prolonged action of the cytotoxic agent.

The entity which is recognised by the target cell-specific portion of the first component of the kit of parts may be any suitable entity which is expressed by tumour cells, virally-infected cells, pathogenic microorganisms, cells introduced as part of gene therapy or normal cells of the body which one wishes to destroy for a particular reason. The entity must be present or accessible to the targeting portion in significantly greater concentrations in or on cells which are to be destroyed than in any normal tissues of the host that cannot be functionally replaced by other therapeutic means.

The entity which is recognised will often be an antigen. Tumour-specific antigens, when they are expressed on the cell membrane or secreted into tumour extra-cellular fluid, lend themselves to the role of targets for antibodies.

The term "tumour" is to be understood as referring to all forms of neoplastic cell growth, including tumours of the lung, liver, skin, pancreas, colon, prostate, uterus or breast. The host is preferably a mammal, most preferably a human, but could in principle be any vertebrate.

The antigen-specific portion may be an entire antibody (usually, for convenience and specificity, a monoclonal antibody), a part or parts thereof (for example an Fab fragment or $F(ab')_2$), a single chain antibody fragment or a synthetic antibody or part thereof. The antibody component may be human or humanised or may be a catalytic antibody. A conjugate comprising only part of an antibody may be advantageous by virtue of better tumour penetration and may be less likely to undergo non-specific binding due to the Fc part. Suitable monoclonal antibodies to selected antigens may be prepared by known techniques, for example those disclosed in "Monoclonal Antibodies: A manual of techniques", H. Zola (CRC Press, 1988) and in "Monoclonal Hybridoma Antibodies: Techniques and Applications", J.G.R. Hurrell (CRC Press, 1982). All references mentioned in this specification are incorporated herein by reference. Bispecific antibodies may be prepared by cell fusion, by reassociation of monovalent fragments or by chemical cross-linking of whole antibodies, with one part of the resulting bispecific antibody being directed to the cell-specific antigen and the other to the enzyme. The bispecific antibody can be administered bound to the enzyme or it can be administered first, followed by the enzyme. Methods for preparing bispecific antibodies are disclosed in Corvalen *et al* (1987) *Cancer Immunol. Immunother.* **24**, 127-132 and 133-137 and 138-143 and Gillsland *et al* (1988) *Proc. Natl. Acad. Sci. USA* **85**, 7719-7723.

The variable heavy (V_H) and variable light (V_L) domains of the antibody are involved in antigen recognition, a fact first recognised by early protease digestion experiments. Further confirmation was found by "humanisation" of

rodent antibodies. Variable domains of rodent origin may be fused to constant domains of human origin such that the resultant antibody retains the antigenic specificity of the rodent parented antibody (Morrison *et al* (1984) *Proc. Natl. Acad. Sci. USA* **81**, 6851-6855).

That antigenic specificity is conferred by variable domains and is independent of the constant domains is known from experiments involving the bacterial expression of antibody fragments, all containing one or more variable domains. These molecules include Fab-like molecules (Better *et al* (1988) *Science* **240**. 1041); Fv molecules (Skerra *et al* (1988) *Science* **240**. 1038); single-chain Fv (ScFv) molecules where the V_H and V_L partner domains are linked via a flexible oligopeptide (Bird *et al* (1988) *Science* **242**. 423; Huston *et al* (1988) *Proc. Natl. Acad. Sci. USA* **85**. 5879) and single domain antibodies (dAbs) comprising isolated V domains (Ward *et al* (1989) *Nature* **341**. 544). A general review of the techniques involved in the synthesis of antibody fragments which retain their specific binding sites is to be found in Winter & Milstein (1991) *Nature* **349**, 293-299.

By "ScFv molecules" we mean molecules wherein the V_H and V_L partner domains are linked via a flexible oligopeptide.

The advantages of using antibody fragments, rather than whole antibodies, are several-fold. The smaller size of the fragments may lead to improved pharmacological properties, such as better penetration of solid tissue. Effector functions of whole antibodies, such as complement binding, are removed. Fab, Fv, ScFv and dAb antibody fragments can all be expressed in and secreted from *E. coli*, thus allowing the facile production of large amounts of the said fragments.

Whole antibodies and $F(ab')_2$ fragments are "bivalent". By "bivalent" we

mean that the said antibodies and $F(ab')_2$ fragments have two antigen combining sites. In contrast, Fab, Fv, ScFv and dAb fragments are monovalent, having only one antigen combining sites. Fragmentation of intact immunoglobulins to produce $F(ab')_2$ fragments is disclosed by Harwood *et al* (1985) *Eur. J. Cancer Clin. Oncol.* **21**, 1515-1522.

IgG class antibodies are preferred.

Alternatively, the entity which is recognised may or may not be antigenic but can be recognised and selectively bound to in some other way. For example, it may be a characteristic cell surface receptor such as the receptor for melanocyte-stimulating hormone (MSH) which is expressed in high numbers in melanoma cells. The cell-specific portion may then be a compound or part thereof which specifically binds to the entity in a non-immune sense, for example as a substrate or analogue thereof for a cell-surface enzyme or as a messenger.

The virus-directed enzyme-pro-drug therapy (VDEPT) approach has been disclosed for the selective killing of neoplastic cells using the transcriptional differences between normal and neoplastic cells to selectively drive expression of enzymes capable of converting a pro-drug into a cytotoxic drug (Huber *et al* (1991) *Proc. Natl. Acad. Sci. USA* **88**, 8039-8043).

A difference in transcription between cells may be associated with tissue-specific promoters, or may be due to changes in activator or repressor molecules in the neoplastic state. Thus in one example, liver-associated albumin transcriptional regulatory sequences may be useful to drive the expression of inhibitor-inactivating enzymes in the treatment of patients with hepatocellular carcinoma. More transcriptional differences between normal and neoplastic cells are being discovered all the time, and it is believed that many

of these differences may be exploited in the methods of the present invention.

Recombinant, replication-defective retroviruses which are suitable for delivering the genetic constructs (ie promoter plus gene encoding inhibitor-inactivating enzyme) to target cells have been disclosed (Huber *et al* (1991) *Proc. Natl. Acad. Sci. USA* **88**, 8039-8043).

Thus, in one embodiment, the first component of the kit may be a system for delivering a suitable genetic construct for VDEPT.

Considerable work has already been carried out on antibodies and fragments thereof to tumour-associated antigens and antibodies or antibody fragments directed at carcinoembryonic antigen (CEA) and antibodies or their fragments directed at human chorionic gonadotrophin (hCG) can be conjugated to carboxypeptidase G2 and the resulting conjugate retains both antigen binding and catalytic function. Following intravenous injection of these conjugates they localise selectively in tumours expressing CEA or hCG respectively. Other antibodies are known to localise in tumours expressing the corresponding antigen. Such tumours may be primary and metastatic colorectal cancer (CEA) and choriocarcinoma (hCG) in human patients or other forms of cancer. Although such antibody-enzyme conjugates may also localise in some normal tissues expressing the respective antigens, antigen expression is more diffuse in normal tissues. Such antibody-enzyme conjugates may be bound to cell membranes via their respective antigens or trapped by antigen in interstitial space.

Examples of cell-specific antigens are given in Table 1.

Table 1

1. Tumour Associated Antigens

Antigen	Antibody	Existing Uses
Carcino-embryonic Antigen	{C46 (Amersham) {85A12 (Unipath)	Imaging & Therapy of colon /rectum tumours.
Placental Alkaline Phosphatase	H17E2 (ICRF, Travers & Bodmer)	Imaging & Therapy of testicular and ovarian cancers.
Pan Carcinoma	NR-LU-10 (NeoRx Corporation)	Imaging & Therapy of various carcinomas incl. small cell lung cancer.
Polymorphic Epithelial Mucin (Human milk fat globule)	HMFG1 (Taylor-Papadimitriou, ICRF)	Imaging & Therapy of ovarian cancer, pleural effusions.
β-human Chorionic Gonadotropin choriocarcinoma	W14	Targeting of enzyme (CPG2) to human xenograft in nude mice (Searle *et al* (1981) *Br.J. Cancer* **44**, 137-144).
A carbohydrate on Human Carcinomas	L6 (IgG2a)1	Targeting of alkaline phosphatase (Senter *et al* (1988) *Proc. Natl. Acad. Sci.*

			USA **85**, 4842-4846
	CD20 Antigen on B Lymphoma (normal and neoplastic)	1F5 (IgG2a)2	Targeting of alkaline phosphatase (Senter *et al* (1988) *Proc. Natl. Acad. Sci.* **85**, 4842-4846

1 Hellström *et al* (1986) *Cancer Res.* **46**, 3917-3923

2 Clarke *et al* (1985) *Proc. Natl. Acad. Sci.* **82**, 1766-1770

Other antigens include alphafoetoprotein, Ca-125 and prostate specific antigen.

2. Immune Cell Antigens

Pan T Lymphocyte Surface Antigen (CD3)	OKT-3 (Ortho)	As anti-rejection therapy for kidney transplants.
B-lymphocyte Surface Antigen (CD22)	RFB4 (Janossy, Royal Free Hospital)	Immunotoxin therapy of B cell lymphoma.
Pan T lymphocyte Surface Antigen (CD5)	H65 (Bodmer, Knowles ICRF, Licensed to Xoma Corp., USA)	Immunotoxin treatment of Acute Graft versus Host Disease, Rheumatoid Arthritis.

3. Infectious Agent-Related Antigens

Mumps virus-related	Anti-mumps polyclonal antibody	Antibody conjugated to Diphtheria toxin for treatment of mumps.
Hepatitis B Surface Antigen	Anti HBs Ag	Immunotoxin against Hepatoma.

It is likely that the enzymatically active portion of the first component will be active in isolation from the cell-specific portion but it is necessary only for it to be enzymatically active when (a) it is in combination with the cell-specific portion and (b) the compound is attached to or adjacent target cells.

It may not be necessary to use a conventional enzyme. Antibodies with catalytic capacity have been developed (Tramontano *et al Science* 234. 1566-1570) and are known as 'abzymes'. These have the potential advantage of being able to be humanized to reduce their immunogenicity.

The two portions of the first component of the kit of parts of the invention may be linked together by any of the conventional ways of cross-linking polypeptides, such as those generally described in O'Sullivan *et al* (1979) *Anal. Biochem.* **100**, 100-108. For example, the antibody portion may be enriched with thiol groups and the enzyme portion reacted with a bifunctional agent capable of reacting with those thiol groups, for example the N-hydroxysuccinimide ester of iodoacetic acid (NHIA) or N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP). Amide and thioether bonds, for example achieved with m-maleimidobenzoyl-N-hydroxysuccinimide ester, are generally more stable *in vivo* than disulphide bonds.

It may not be necessary for the whole enzyme to be present in the first component (or the third component, if the third component metabolises the drug) of the kit of parts but, of course, the catalytic portion must be present.

Alternatively, said first component may be produced as a fusion compound by recombinant DNA techniques whereby a length of DNA comprises respective regions encoding the two portions of the compound of the invention either adjacent one another or separated by a region encoding a linker peptide which does not destroy the desired properties of the compound. Conceivably, the two portions of the compound may overlap wholly or partly.

The DNA is then expressed in a suitable host to produce a polypeptide comprising the compound of the invention. Thus, the DNA encoding the polypeptide constituting the compound of the invention may be used in accordance with known techniques, appropriately modified in view of the teachings contained herein, to construct an expression vector, which is then used to transform an appropriate host cell for the expression and production of the polypeptide of the invention. Such techniques include those disclosed in US Patent Nos. 4,440,859 issued 3 April 1984 to Rutter *et al*, 4,530,901 issued 23 July 1985 to Weissman, 4,582,800 issued 15 April 1986 to Crowl, 4,677,063 issued 30 June 1987 to Mark *et al*, 4,678,751 issued 7 July 1987 to Goeddel, 4,704,362 issued 3 November 1987 to Itakura *et al*, 4,710,463 issued 1 December 1987 to Murray, 4,757,006 issued 12 July 1988 to Toole, Jr. *et al*, 4,766,075 issued 23 August 1988 to Goeddel *et al* and 4,810,648 issued 7 March 1989 to Stalker, all of which are incorporated herein by reference.

The DNA encoding the polypeptide constituting the compound of the invention may be joined to a wide variety of other DNA sequences for introduction into an appropriate host. The companion DNA will depend upon the nature of the host, the manner of the introduction of the DNA into the host, and whether

episomal maintenance or integration is desired.

Generally, the DNA is inserted into an expression vector, such as a plasmid, in proper orientation and correct reading frame for expression. If necessary, the DNA may be linked to the appropriate transcriptional and translational regulatory control nucleotide sequences recognised by the desired host, although such controls are generally available in the expression vector. The vector is then introduced into the host through standard techniques. Generally, not all of the hosts will be transformed by the vector. Therefore, it will be necessary to select for transformed host cells. One selection technique involves incorporating into the expression vector a DNA sequence, with any necessary control elements, that codes for a selectable trait in the transformed cell, such as antibiotic resistance. Alternatively, the gene for such selectable trait can be on another vector, which is used to co-transform the desired host cell.

Host cells that have been transformed by the recombinant DNA of the invention are then cultured for a sufficient time and under appropriate conditions known to those skilled in the art in view of the teachings disclosed herein to permit the expression of the polypeptide, which can then be recovered.

Many expression systems are known, including bacteria (for example *E. coli* and *Bacillus subtilis*), yeasts (for example *Saccharomyces cerevisiae*), filamentous fungi (for example *Aspergillus*), plant cells, animal cells and insect cells.

The vectors include a procaryotic replicon, such as the ColE1 *ori*, for propagation in a procaryote, even if the vector is to be used for expression in other, non-procaryotic, cell types. The vectors can also include an appropriate promoter such as a procaryotic promoter capable of directing the expression (transcription and translation) of the genes in a bacterial host cell, such as *E.*

coli, transformed therewith.

A promoter is an expression control element formed by a DNA sequence that permits binding of RNA polymerase and transcription to occur. Promoter sequences compatible with exemplary bacterial hosts are typically provided in plasmid vectors containing convenient restriction sites for insertion of a DNA segment of the present invention.

Typical procaryotic vector plasmids are pUC18, pUC19, pBR322 and pBR329 available from Biorad Laboratories, (Richmond, CA, USA) and p*Trc*99A and pKK223-3 available from Pharmacia, Piscataway, NJ, USA.

A typical mammalian cell vector plasmid is pSVL available from Pharmacia, Piscataway, NJ, USA. This vector uses the SV40 late promoter to drive expression of cloned genes, the highest level of expression being found in T antigen-producing cells, such as COS-1 cells.

An example of an inducible mammalian expression vector is pMSG, also available from Pharmacia. This vector uses the glucocorticoid-inducible promoter of the mouse mammary tumour virus long terminal repeat to drive expression of the cloned gene.

Useful yeast plasmid vectors are pRS403-406 and pRS413-416 and are generally available from Stratagene Cloning Systems, La Jolla, CA 92037, USA. Plasmids pRS403, pRS404, pRS405 and pRS406 are Yeast Integrating plasmids (YIps) and incorporate the yeast selectable markers *his3*, *trp1*, *leu2* and *ura3*. Plasmids pRS413-416 are Yeast Centromere plasmids (YCps)

A variety of methods have been developed to operatively link DNA to vectors via complementary cohesive termini. For instance, complementary

homopolymer tracts can be added to the DNA segment to be inserted to the vector DNA. The vector and DNA segment are then joined by hydrogen bonding between the complementary homopolymeric tails to form recombinant DNA molecules.

Synthetic linkers containing one or more restriction sites provide an alternative method of joining the DNA segment to vectors. The DNA segment, generated by endonuclease restriction digestion as described earlier, is treated with bacteriophage T4 DNA polymerase or *E. coli* DNA polymerase I, enzymes that remove protruding, 3'-single-stranded termini with their 3'-5'-exonucleolytic activities, and fill in recessed 3'-ends with their polymerizing activities.

The combination of these activities therefore generates blunt-ended DNA segments. The blunt-ended segments are then incubated with a large molar excess of linker molecules in the presence of an enzyme that is able to catalyze the ligation of blunt-ended DNA molecules, such as bacteriophage T4 DNA ligase. Thus, the products of the reaction are DNA segments carrying polymeric linker sequences at their ends. These DNA segments are then cleaved with the appropriate restriction enzyme and ligated to an expression vector that has been cleaved with an enzyme that produces termini compatible with those of the DNA segment.

Synthetic linkers containing a variety of restriction endonuclease sites are commercially available from a number of sources including International Biotechnologies Inc, New Haven, CN, USA.

A desirable way to modify the DNA encoding the polypeptide of the invention is to use the polymerase chain reaction as disclosed by Saiki *et al* (1988) *Science* **239**, 487-491.

In this method the DNA to be enzymatically amplified is flanked by two specific oligonucleotide primers which themselves become incorporated into the amplified DNA. The said specific primers may contain restriction endonuclease recognition sites which can be used for cloning into expression vectors using methods known in the art.

Exemplary genera of yeast contemplated to be useful in the practice of the present invention are *Pichia*, *Saccharomyces*, *Kluyveromyces*, *Candida*, *Torulopsis*, *Hansenula*, *Schizosaccharomyces*, *Citeromyces*, *Pachysolen*, *Debaromyces*, *Metschunikowia*, *Rhodosporidium*, *Leucosporidium*, *Botryoascus*, *Sporidiobolus*, *Endomycopsis*, and the like. Preferred genera are those selected from the group consisting of *Pichia*, *Saccharomyces*, *Kluyveromyces*, *Yarrowia* and *Hansenula*. Examples of *Saccharomyces* are *Saccharomyces cerevisiae*, *Saccharomyces italicus* and *Saccharomyces rouxii*. Examples of *Kluyveromyces* are *Kluyveromyces fragilis* and *Kluyveromyces lactis*. Examples of *Hansenula* are *Hansenula polymorpha*, *Hansenula anomala* and *Hansenula capsulata*. *Yarrowia lipolytica* is an example of a suitable *Yarrowia* species.

Methods for the transformation of *S. cerevisiae* are taught generally in EP 251 744, EP 258 067 and WO 90/01063, all of which are incorporated herein by reference.

Suitable promoters for *S. cerevisiae* include those associated with the *PGK1* gene, *GAL1* or *GAL10* genes, *CYC1*, *PHO5*, *TRP1*, *ADH1*, *ADH2*, the genes for glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, triose phosphate isomerase, phosphoglucose isomerase, glucokinase, α-mating factor pheromone, a-mating factor pheromone, the *PRB1* promoter, the *GUT2* promoter, and hybrid promoters involving hybrids of parts of 5' regulatory regions with parts of 5' regulatory regions of other promoters or with upstream activation sites (eg the

promoter of EP-A-258 067).

The transcription termination signal is preferably the 3' flanking sequence of a eukaryotic gene which contains proper signals for transcription termination and polyadenylation. Suitable 3' flanking sequences may, for example, be those of the gene naturally linked to the expression control sequence used, ie may correspond to the promoter. Alternatively, they may be different in which case the termination signal of the *S. cerevisiae AHD1* gene is preferred.

The second component of the kit is a pro-drug which is relatively non-toxic, which is a substrate for the enzyme in the first component of the kit and which is converted to a cytotoxic substance. The cytotoxic substance may be any existing anti cancer drug such as an alkylating agent, an agent which intercalates in DNA, inhibits any key enzymes such as dihydrofolate reductase, thymidine synthetase, ribonucleotide reductase, nucleoside kinases or topoisomerase or effects cell death by interacting with any other cellular constituent. Etoposide is an example of a topoisomerase inhibitor.

It is evident that the inactivating portion of the compound of the invention which forms the third component of the kit of parts must be chosen to suit the active drug that has been generated. It is also necessary for most of the inactivation of the cytotoxic drug to be confined to the vascular compartment so as to avoid undesirable inactivation of the active drug at target sites.

It will also be appreciated that the inactivating portion of the compound does not inactivate the prodrug which may also be present in the blood, in such a way that its conversion into the cytotoxic drug is prevented. It is desirable to have prodrug in the blood so that it can reach tumour sites, where it is converted to the cytotoxic drug. In accordance with preferred embodiments of the present invention a means is therefore provided by which an active drug

generated from a prodrug at particular sites in the body can be inactivated in the blood of a patient without comparable inactivation of any prodrug which is present in the blood.

The inactivating portion may be an enzymatically active portion, capable of converting the active drug into a less toxic substance. It may not be necessary for the whole enzyme to be present but, of course, the catalytic portion must be present. In the case of methotrexate as the active drug, for example, the inactivating portion may be the enzyme carboxypeptidase G2 or another folate-deglutamating enzyme, which degrades methotrexate thereby inactivating it.

The bacterial enzymes carboxypeptidase G1 and G2 (CPG1 and CPG2) degrade folates including methotrexate by cleavage of the terminal glutamic acid. The actions of the two enzymes are thought to be the same. The following description of preferred aspects of the invention refers to CPG2 but is equally applicable to CPG1 and to any other enzymes acting on the same substrates, and to abzymes acting on the same substrates.

The isolation, purification and some of the properties of carboxypeptidase G2 from *Pseudomonas sp.* strain RS-16 have been disclosed by Sherwood *et al* (1984) *Eur. J. Biochem.* **148**, 447-453. The cloning of the gene encoding the said carboxypeptidase G2, its nucleotide sequence and its expression in *E. coli* have been disclosed by Minton *et al* (1984) *Gene* **31**, 31-38 and Minton *et al* (1983) *J. Bacteriol.* **156**, 1222-1227. CP2G2 is available from the Division of Biotechnology, Centre for Applied Microbiological Research, Porton Down, Salisbury, UK. Carboxypeptidase G1 (CPG1) is disclosed by Chabner *et al* (1972) *Cancer Res.* **32**, 2114-2119.

The inactivating portion may alternatively be a chemical moiety which reacts spontaneously with the active cytotoxic drug or does so more effectively in the

presence of an appropriate catalyst. For example, in the case of an alkylating agent as the drug, the inactivating portion may comprise a thiol-containing substance and inactivation is catalysed by glutathione transferase carried in or on macromolecular entities.

The inactivating portion may alternatively, for example, be an antibody or part thereof which binds to the active drug, but not to the corresponding prodrug, or does so only to a more limited extent, or which is catalytically active towards the pro-drug thereby inactivating it, but not active towards the drug.

The production of antibodies to low molecular weight compounds such as cytotoxic drugs may be facilitated by the well known technique of haptenisation, in which the low molecular weight molecule is conjugated to a highly immunogenic protein, such as keyhole limpet haemocyanin or other carrier molecule.

In situations where prodrug is converted to active drug by catalytic cleavage of a moiety of the prodrug, the production of an antibody capable of discriminating between prodrug and active drug is favoured since, in the prodrug, the non-active moiety can sterically inhibit binding of an anti-drug antibody to the thus hidden drug portion of the prodrug.

The inactivating portion may be conjugated to the restraining portion according to methods of linkage known in the art, thereby to retain the compound of the invention in the vascular compartment.

A further component may achieve accelerated clearance of antibody-enzyme conjugate and/or inactivation of the specific enzyme from non-tumour sites. Several means by which this can be achieved have been described (PCT Patent Application WO 89/10140 & Bagshawe, 1989). For example, in one method,

an antibody directed at the enzyme is employed. To prevent such an antibody from combining with and possibly inactivating enzyme at tumour sites additional galactose residues are added which ensure that this second antibody and antibody-enzyme conjugate are quickly cleared from the blood by binding to galactose receptor rich hepatocytes.

The antibody used for clearing or inactivating the antibody-enzyme conjugate can be directed towards the antigen binding site on the antitumour antibody, or the active site of the enzyme, or any other site on the antibody-enzyme conjugate. Such antibodies may have additional galactose residues or other sugars added to accelerate clearance or may be desialylated. Galactosylation of the antibody results in its rapid clearance from the blood through take-up by galactose receptors on hepatocytes. Alternatively, or additonally, the antibody-enzyme conjugate is galactosylated, and given after the hepatic galactose receptors have been blocked by asialo-bovine submaxillary gland mucoprotein or antibody directed at hepatic galactose receptor or other molecule with high affinity for galactose receptor. The blocking substance is maintained in plasma for a period of up to 24 hours so that the antibody-enzyme complex localises at tumour sites but following cessation of galactose receptor blockade, the galactosylated antibody-enzyme is quickly cleared via the available galactose receptors.

A component may also be required to avoid the constraints which could be imposed by the host immune response to foreign proteins. The nature of this component may change with the stage of development of methods of immunological control. Methods which can be used to overcome the host response to foreign protein are known in the art. Techniques for reducing the immunogenicity of foreign proteins, applicable to antibody-enzyme conjugates, is that of conjugation to forms of polyethylene glycol (Wilkinson *et al* (1987) *J. Immunol.* **139**, 326-331).

Alternatively, or additionally, the problem of immunogenicity may be overcome by administering immunosuppressors or immune tolerance inducing agents. Cyclosporin and FK506 are widely used drugs to achieve immunosuppression in tissue transplantation. Cyclosporin has been shown to delay host antibody respone to foreign protein (Lederman *et al* (1988) *Br. J. Cancer* 58, 562-566 and 654-657). Tolerance to foreign proteins when the host encounters the foreign problem for the first time after receiving an antibody directed at the CD4 epitope on lymphocytes has been disclosed (Waldman *et al* (1988) pp 16-30 in *Progress in Allergy* (Shizata & Woksman. Eds, New York). Further means to achieve this have been described elsewhere and may change as improvements occur in control of host antibody responses to foreign antigens. Catalytic antibodies (abzymes) may be 'humanized' to reduce or remove their immunogenicity.

A fourth aspect of the present invention provides a method of destroying target cells in a host, the method comprising administration to the host of the various components described above.

The components of the invention are administered in any suitable way, usually parenterally, for example intravenously, intraperitoneally or intravesically, in standard sterile, non-pyrogenic formulations of diluents and carriers.

When an antibody directed at a tumour associated antigen or an antibody-enzyme conjugate is injected into an appropriate tumour bearing host, only a small fraction of the antibody or conjugate localises at the tumour site and most of it remains in blood and other normal tissues for several days. Thus, although tumour concentration of the enzyme will be higher than in normal tissues, the volume of normal tissues is much greater. Thus, to minimize the amount of enzyme residual in normal tissues and blood it may be desirable to use the methods of the present invention in conjunction with the methods

disclosed in WO 89/10140; Bagshawe (1989) *Brit. J. Cancer* **60**, 275-281; and Sharma *et al* (1990) *Brit. J. Cancer* **61**, 659-662 for inactivating and clearing excess antibody-enzyme conjugate from the blood.

In attempting to achieve eradication of cancers it may not be possible to avoid suppression of haemopoietic function (myelosuppression) although for a given effect on a tumour target it is expected to be much less with the system described herein. Similarly, for a given degree of myelosuppression a much greater tumour effect is expected. Growth factors or growth inhibitory factors acting on haemopoietic tissues may therefore be usefully employed in combination with the system described herein.

The system described herein may be used in conjunction with other forms of therapy. These include conventional cytotoxic agents, and use of multiple enzyme delivery to inactivate more than one metabolite.

Similarly, an enzyme delivered to tumour sites by an antibody may function both to activate a pro-drug and to inactivate a metabolite which protects normal tissues. Carboxypeptidase G2 as disclosed herein inactivates folinic acid at tumour sites to leave the tumour cells unprotected against trimetrexate. The same tumour located enzyme can activate a benzoic acid pro-drug to form a cytotoxic mustard (as disclosed by Bagshawe (1989) *Brit. J. Cancer* **60**, 275-281).

The compounds and methods of the present invention will be discussed in the following Examples and Figures with specific references to cytotoxic therapy utilising the agent methotrexate, which is inactivated by the enzyme carboxypeptidase G2. The agent aminopterin is another powerful cytotoxic agent which is inactivated by the enzyme carboxypeptidase G2.

Figure 1 shows the structures of methotrexate and folinic acid.

Figure 2 shows the structures of (I) a pro-drug and (II) the cytotoxic drug (a benzoic acid mustard) produced after cleavage of (I) with carboxypeptidase G2.

Figure 3 shows a scheme for conjugating aminobenzoic acid to keyhole limpet haemocyanin.

Figure 4 is a diagrammatic representation of the invention wherein the first component is a target-cell specific antibody coupled to carboxypeptidase A (CPA); the second component is alanine-methotrexate; and the third component is a restraining portion coupled to carboxypeptidase G2 (CPG2).

The invention relates to a general principle and may be applied to other cytotoxic agents.

Example 1: A therapeutic system using Ala-methotrexate carboxypeptidases A and G2

The widely used agent methotrexate is a folic acid antagonist and its action is to block the conversion of folic acid, a dietary factor, to its reduced form 5-methyltetrahydrofolic acid (folinic acid, citrovorum factor) by the enzyme dihydrofolate reductase. Folinic acid is used in one carbon transfer in the synthesis of DNA. In the absence of folinic acid, cell reproduction is blocked in S phase and cell death follows. Methotrexate is used in the treatment of a wide range of malignant diseases. It also causes cell death in normal cell renewal tissues via the mechanisms already outlined. The magnitude of its effects is largely a function of the duration of tissue exposure to the drug, the longer the duration the greater the toxic effect. Susceptibility to the action of methotrexate is thought to result from polyglutamation of the drug which, by

delaying its breakdown within and its excretion from the cell, favours prolonged action. The effect of methotrexate can be by-passed by folinic acid, generally given in the form of 5-formyl tetrahydrofolic acid. Carefully timed and dose-controlled administration of methotrexate with folinic acid has been found advantageous over the use of methotrexate alone in the treatment of some cancers. Thus large doses of methotrexate are commonly followed after 12-24 hours by folinic acid 'rescue'. Similarly, administration of low dose methotrexate on alternate days and folinic acid on each succeeding day has proved to be a successful and low toxicity treatment for many patients with some forms of trophoblastic tumour (Bagshawe *et al* (1989) *Brit. J. Ob. Gynae* **96**. 795-802).

It has been shown (Kuefner *et al* (1989) *Biochemistry* **28**, 2288-2297) that when methotrexate is modified by introducing an alanine moiety via an amide linkage to the α carboxyl of methotrexate the resulting compound is effectively excluded from cells and the toxicity of the alanine form is 50-100 fold less than that of native methotrexate in target cells *in vitro*. The alanine moiety is cleaved by the action of the enzyme carboxypeptidase A leaving native methotrexate. Alanine methotrexate is not metabolised by the enzyme carboxypeptidase G2. Carboxypeptidase G2 degrades methotrexate and natural folates by cleavage of the glutamic acid moiety.

In the present example, the first component of the system of the present invention comprises an antibody or antibody fragment conjugated to carboxypeptidase A, directed at the tumour associated antigen, carcinoembryonic antigen.

Of course, a carboxypeptidase other than carboxypeptidase A but with the same substrate specificity may be used in place of carboxypeptidase A.

Carboxypeptidase A is available from bovine and bacterial sources (for example from Calbiochem, Nottingham, UK) and is also present in human pancreas. The enzyme hydrolyses oligopeptides from the C terminal end of polypeptide chains, or from other compounds containing conjugated amino acids with a free carboxyl group. Carboxypeptidase A has a preference for aromatic residues. It is normally formed from mammalian sources by trypsinization of a complex assembly of three subunits produced by the pancreas.

A further component may achieve accelerated clearance or inactivation of carboxypeptidase A from non-tumour sites.

The second component is alanine methotrexate (Kuefner *et al* 1989) which is the prodrug from which the active drug methotrexate is generated by the action of carboxypeptidase A.

The third component is carboxypeptidase G2 conjugated to a macromolecular structure such as dextran. The purpose of the macromolecule is to confine CPG2 activity to the vascular compartment.

For example, CPG2 may be coupled to soluble dextrans (Lomodex 40. Lomodex 70, Dextraven 110 and Dextraven 150. all trade marks: Fisons. Loughborough, Leics., UK) according to the method of Melton *et al* (1987). A volume of dextran prepartion containing 1 g of dextran in 0.9% NaCl was diluted to 100 ml with 0.9% NaCl and reacted with cyanogen bromide (CNBr: Sigma, Poole, Dorset, UK). CNBr (0.5 g) was used for activating the 40- and 70,000 dalton dextrans and 0.4 g for the higher molecular weight dextrans. This reduction was necessary to prevent precipitation of the 110.000 and 150,000 dalton dextrans. The reaction mixture was vigorously stirred at room temperature and maintained at pH 10.7 $\pm$ 0.1 units in a pH-stat (Radiometer. Copenhagen, Denmark) by addition of 2 M NaOH. The CNBr was added as

a finely divided powder in two equal portions at an interval of 20 min; the second portion was allowed to react until the pH of the reaction mixture was stable at 10.7; the pH was then adjusted to 9.0 and the mixture dialysed against running water for 2 hr at 4°C. The pH was brought back to 9.0 with 1 M NaOH and 1 ml enzyme solution (1265 U; 2.3 mg) in 0.1 M Tris-HCl buffer, pH 7.3, was added. The mixture was reacted overnight at 4°C after which 0.25 g glycine was added to block excess reactive sites. The mixture was stirred for a further 30 min and then concentrated to a volume of 40 ml in a model 202 concentrator using a PM10 ultrafiltration membrane (Amicon, Stonehouse, UK). The mixture (40 ml) was then chromatographed on a 1.3 litre bed volume of Sephadex G150 in a 4.4 x 87 cm column (Pharmacia, Uppsala, Sweden) and eluted with 0.05 M potassium phosphate buffer, pH 7.0. Fractions (10 ml) were collected and assayed for enzyme activity; carbohydrate content was determined by the phenol-sulphuric acid method (M. Dubois *et al* (1956) *Analyt. Chem.* **28**, 350) using dextran-70 as standard in the range 0-100 μg/ml (Sephadex is a trade mark).

The peak fractions were pooled and concentrated to a volume of 10-12 ml as before. Enzyme activity and carbohydrate content were determined and protein content measured by the Coomassie blue method (M.M. Bradford (1976) *Analyt. Biochem.* **72**, 248) using bovine serum albumin fraction V as standard in the range 0-100 μg/ml. The concentrated material was filter sterilised (Millipore "Millex GS", 0.22 μm pore size) and stored at -20°C. Millex GS is a trade mark.

The antibody-carboxypeptidase A conjugate, if comprising antibody of murine origin and enzyme of bovine origin, would be immunogenic. Similarly, a carboxypeptidase G2 macromolecule conjugate would also be immunogenic since CPG2 is bacterial in origin. It may be desirable, therefore, to reduce their immunogenicity or to employ means to induce immunosuppression or

immune tolerance.

Example 2: Method of use

Administration of a component capable of overcoming the host response to foreign protein may be started 48 hours before administration of Component 1. Initial tests may be performed to exclude as far as possible abnormal reaction by the patient to any of the protein components. The antibody-CPA conjugate is given intravenously, preferably by slow infusion, typically over 2 hours. Maximal tumour concentration of the antibody-CPA conjugate is achieved several hours later but at this time there are still high levels of CPA activity in plasma. It is desirable to eliminate this activity as far as possible before administering the prodrug. This elimination process is achieved by administration of a component capable of achieving accelerated clearance or inactivation of CPA from non-tumour sites. This component is administered intravenously over several hours, typically 6-24 hours, or until enzyme is no longer detectable in plasma and may be infused at low concentration throughout the period of pro-drug administration. During this time enzyme in extracellular fluid diffuses back into the plasma as the plasma level of the enzyme falls. Tests for enzyme activity from plasma are continued for a period typically 8-24 hours to ensure that plasma CPA activity is not detectable. More of said component which eliminates the CPA activity is given if necessary. Alternative methods of accelerated clearance have been described.

Administering component 3 is then commenced either by a series of bolus injections or by slow infusion.

It is preferred that components 2 and 3 are started simultaneously (ie the pro-drug and the inactivating compound).

Pro-drug may be given as a series of bolus injections or by continuous infusion. Administration of components 2 and 3 will normally continue for about 4-7 days but specific embodiments of Component 1 may ensure that sufficient enzyme actively persists at tumour sites for somewhat longer periods.

At about 7-10 days after administration of component it is desirable to review enzyme activity at tumour sites. Administration of the component capable of overcoming the host response to foreign protein is continued, component 2 is discontinued, component 3 may be discontinued.

Component 1 is reinfused as previously, followed by the component which eliminates the plasma CPA activity as previously. Similar procedures to those previously described are followed before recommencing component 2.

The cycle may be repeated. Limiting factors will be toxicity attributable to alanine methotrexate or the development of host antibodies to any of the foreign proteins employed.

Example 3: Use of quinazoline antifolates

A similar system to that disclosed in Examples 1 and 2 can be used with at least some members of a series quinazoline antifolates which have been described (Jones *et al* (1986) *J. Med. Chem.* **29**, 468-472; Jodrell *et al* (1991) *Brit. J. Cancer* **64**, 833-8; Harrap *et al* (1989) *Advances in enzyme regulation* **29**, 161; Jackman *et al* (1991) *Advances Enzyme Regulat.* **31**, 13; Jodrell *et al* (1990) *Proc. Am. Assoc. Cancer Res.* **31**, 341. These agents differ from natural folates and methotrexate with respect to substitution for instance at the N^{10} position and in the benzoyl ring but like natural folates and methotrexate have a terminal glutamate moiety linked to the benzoyl ring. Therefore at least some of the drugs in this series are inactivated by a peptide substitution such

as an alanine in the α position of the glutamate, and that such alanine- or other peptide-substituted derivatives are synthesized using the methodology described by Kuefner *et al loc. cit.* or variations thereof known in the art. Similarly, such quinazoline antifolates are deglutamated, and therefore inactivated, by carboxypeptidase G2 or a similar enzyme. These compounds are of particular interest because they act by inhibition of thymidylate synthetase. The chemical application of such drugs may be greatly extended by being administered in pro-drug form. converted to the active compound by carboxypeptidase A and the active drug in plasma degraded by carboxypeptide G2.

Example 4: Production of antibodies discriminating between active drug and pro-drug

In order to raise an antibody that would recognise the active drug (II) but not the pro-drug (I), a compound was synthesised which represented the region of greatest divergence between the two molecules (I) and (II) (Figure 3). This region corresponds to the acid portion of the benzoic acid mustard drug (II). Since benzoic acid itself is not large enough to be immunogenic. it was considered that the most effective method of raising antibodies specific for the acid region would be to inoculate animals with a benzoic acid analogue that had been previously conjugated to Keyhole Limpet Haemocyanin (KLH). A compound was synthesised that consisted of an L-lysine amino acid linked through an amide bond to aminobenzoic acid (VII). Then (VII) was conjugated to KLH by conventional methods using the ϵ-NH_2 groups on the lysine portion of the molecule, to produce the specific immunogen (VIII).

1. Preparation of (IV)

The Nα,Nε-di-tert-Benzyloxycarbonyl-L-lysine (IV) was liberated from its dicyclohexylammonium salt (III). Briefly, (III) (4 mmol) was suspended in

ethyl acetate (100 ml), then washed with cold citric acid (10%). The organic layer was separated, dried over sodium sulphate and evaporated to dryness to (IV), a white gum. Yield 100%. NMR (Me_2SO-δ_6) δ1.4 (bd.s. 22 H), 1.6 (m. 2H), 2.9 (d, 2H), 3.8 (m, 1H), 6.7 (m. 1H), 7.0 (d. 1H).

2. Preparation of (VI)

Compound (IV) was coupled to 4-aminobenzoyl tert-butyl ester (V) by a modification of a peptide coupling literature method ((1989) *J. Med. Chem.* 31. 163). To a stirred solution of (IV) (2 mmol) and N-methylmorpholine (2 mmol) in THF (2 ml), cooled to -20°C. was added isobutyl chloroformate (2 mmol). After 10 minutes. a suspension of (V) (2 mmol) in THF (2 ml) containing N-methylmorpholine (2 mmol) was added. The stirring was continued for 10 minutes at -20°C. and then the mixture allowed to warm to room temperature. The N-methylmorpholine hydrochloride was filtered off and the filtrate evaporated to dryness. The resulting crude mixture was twice chromatographed on silica gel to give the novel pure product as an orange oil (VI). Yield 4%. NMR (Me_2SO-δ_6) δ 1.36 (s. 9H). 1.38 (s. 13H). 1.54 (s. 9H). 1.61 (m. 2H). 2.90 (d. 2H). 4.02 (m. 1H). 6.73 (m. 1H). 7.00 (d. 1H). 7.71 (d, 2H), 7.85 (d. 2H). 10.22 (s. 1H) Mass Spectrum m/z 521 (M).

3. Preparation of (VII)

The deprotection of compound (VI) (0.1 mmol) was effected by suspension in TFA (2 mL). After 40 minutes. the solution was evaporated to dryness to give the novel product (VII) as a white solid. Yield 100%. NMR (Me_2SO-δ_6) δ 1.40 (m, 2H), 1.55 (m, 2H), 1.82 (m. 2H), 2.76 (m. 2H). 3.97 (m. 1H). 7.65 (bd.s, 2H), 7.73 (d, 2H), 7.97 (d, 2H), 8.27 (bd.s. 2H). 10.75 (s. 1H) Mass Spectrum (FAB) m/z 265 ([M + H^+])

4. Preparation of (VIII)

The coupling of compound (VII) to keyhole limpet haemocyanin (KLH) was effected by a modification of the literature method of Hancock and Evan ((1992) *Methods in Molecular Biology* 10, 23). Briefly, (VII) was added to KLH (equivalent weight for weight) and the mixture adjusted with sodium bicarbonate and glutaraldehyde to a final concentration of 0.1M and 0.05% respectively. The mixture was stirred for 24 hours before the addition of glycine ethyl ester to a final concentration of 0.1M. The reaction mixture was left 30 minutes. before the addition of cold acetone (36 ml). After a further 30 minutes. the precipitated conjugate was centrifuged, the supernatant removed and the pellet air-dried to yield a pink solid. The conjugate was stored at -20°C prior to inoculation.

5. Preparation of (VIII) for inoculation

The conjugate was suspended in saline (0.9%) to a final concentration of 1 mg/ml. The suspension was then emulsified in Freund's adjuvant (Complete or Incomplete), prior to inoculation.

Polyclonal and monoclonal antibodies were raised to the conjugate using methods well known in the art.

In the case of the monoclonal antibodies the supernatant from clones of hybridoma cells grown under conventional conditions was tested for binding to pro-drug and its cytotoxic derivative. Most clones reacted with both pro-drug and active drug. The supernatant from two clones bound only to active drug.

Example 5: Use with ADEPT

The ADEPT concept uses an antibody-enzyme conjugate to generate a cytotoxic drug from an inactive precursor at tumour sites. The present invention is used in conjuction with ADEPT. Active drug is generated at the tumour site using the ADEPT antibody-enzyme conjugate. Any active drug entering the blood compartment is cleared from the blood using the inactivating agent coupled to the restraining portion.

In the case of ADEPT treatment, nude mice bearing human choriocarcinoma (CC3) tumours received 29 units of CPG2 conjugated to anti-HCG (W14 Fab_2, as disclosed in WO 88/07378), and after 24 or 48 hours received pro-drug (41 μM/kg). The amount of inactivating agent is adjusted to give an optimal protective effect.

Example 6: Preparation of a monoclonal antibody reactive against carcinoembryonic antigen

Purified CEA was prepared from metastases of colonic tumour. Radio-iodination to a specific activity of 6 $\mu Ci\mu g^{-1}$ was carried out by the iodogen method. Dilution buffer was prepared as a 0.15M sodium phosphate buffer, pH 7.4, containing 0.1% bovine serum albumin. The studies at low ionic strength were carried out in 0.02M Tris-HCl buffer at pH 7.4.

Immunisation schedule: Monoclonal antibody A_5B_7 was raised against purified, heat-treated CEA using the following procedure. One milligram of purified CEA was heated at 85°C for 35 min in 0.05 M phosphate buffer (pH 7) at a concentration of 1 mg ml^{-1}. After mixing with 1 ml of 10% aqueous potassium aluminium sulphate (alum), the pH was adjusted with constant stirring to 6.5-7 by dropwise addition of NaOH solution. After stirring at room

temperature for 30 min the resulting precipitate was washed three times in saline. It was then mixed with 10^{10} formalised *Bordetella pertussis* (kindly supplied by Wellcome Research Laboratories). Three different immunisation schedules were used.

Spleen cells from the immunised mice were then fused with either SP2/0-Ag 14 or P3-NS/1-Ag 4-1 myeloma cells (Flow Laboratories. UK) and the hybridomas producing anti-CEA cloned by single cell transfer.

Example 7: Preparation of F(ab')$_2$ fragments of A_5B_7

The monoclonal anti-CEA (A_5B_7) used in this study has been described previously and chosen for its low cross-reactivity with NCA and its stability on immunopurification and radiolabelling. F(ab')$_2$ fragments were prepared by the method Lamoyi and Nisonoff (1983) *J. Immunol. Methods* 56. 235-243. After separation of the digest mixture on Sephacryl S-200, the fractions were analysed by SDS-PAGE using a 7.5% gel. The fraction containing the F(ab')$_2$ was concentrated and dialysed against 0.15M phosphate buffer. pH 7. Both intact A_5B_7 and the fragment were shown to be immunologically active and relatively homogeneous by electroblotting of the SDS gel onto nictrocellulose paper and overlaying with ^{125}I-labelled CEA. Intact A_5B_7 and its F(ab')$_2$ fragment were radiolabelled by the chloramine T method to specific activities of 5.6 and 5.2 μCi/μg respectively. Both labelled preparations were shown to retain immunological activity by solid-phase radioimmunoassay using CEA coupled to amino-cellulose (Rogers *et al* (1983) *Eur. J. Cancer Clin. Oncol.* **19**, 629-639). An excess of 60% activity was retained in each case.

Example 8: Production of a monoclonal antibody reactive against carboxypeptidase A

A monoclonal antibody raised against carboxypeptidase is used for making bispecific antibody (see next Example) and for clearance and inactivation of residual enzyme activity at non-tumour sites.

The monoclonal antibody was made in the following way. Balb/C mice (6-8 weeks old) were immunised with 50 μg CPA i.p. in incomplete Freund's adjuvant followed by two injections of CPA in complete Freund's adjuvant (50 μg CPA each, i.p.) at monthly intervals and with two daily injections (50 μg and 100 μg in PBS, i.v.) 2 days before fusion. Immune spleen cells were fused with non-immunoglobulin secreting SP2/0 myeloma cells according to the hybridoma procedures of Köhler and Milstein (1975).

The presence of anti-CPA antibodies was detected by a solid-phase indirect radioimmunoassay. A 1 μg ml^{-1} solution of CPA in 0.05 M phosphate buffer was placed in polyvinyl microtitre plates (100 ng per well), allowed to dry, fixed with methanol and washed with PBS buffer containing a 0.05% Tween and 0.1% bovine serum albumin. Supernatant or purified antibody samples were diluted in PBS and incubated in the CPA coated microtitre plates (100 μl per well) at 37°C for 4 h and then for 1 h with ^{125}I-labelled rabbit anti-mouse IgG. The wells were washed three times with PBS-Tween buffer between each stage and after final washing individual wells were cut and counted in a gamma counter.

Example 9: Bispecific antibody reactive against CPA and CEA

The hybridoma producing A_5B_7, a monoclonal antibody reactive against CEA has been disclosed by Harwood *et al* (1986) *Brit. J. Cancer* **54**, 75-82, and a

method of generating a hybridoma, a monoclonal antibody reactive against CPA is disclosed in Example 8.

The fusion protocol allows any two antibody-producing hybridomas to be fused and has been diclosed previously (Clark & Waldmann (1987) *J. Natl. Cancer Inst.* **79**, 1393-1401). Briefly, 5 x 10^6 to 3.5 x 10^7 cells of one parental hybridoma that have been previously renderedhypoxanthine/aminopterin/-thymidine (HAT) sensitive by selection for a hypoxanthine phosphoribosyltransferase-negative variant were fused at 1 1:1 or 10:1 ratio. using 1 ml of a 50% (wt/vol) solution of polyethylene glycol. with the second parental hybridoma cells that had been pretreated with a lethal dose of 10 mM iodoacetamide. Excess polyethylene glycol was washed out and the cells were plated at concentrations from 8 x 10^5 per ml to 2 x 10^5 per ml into 24-well plates in bicarbonate-buffered Iscove's modified Dulbecco's medium (IMDM) supplemented with 5% (vol/vol) fetal calf serum. After 24 hr in culture. hybrid hybridomas were selected for in medium containing HAT.

Example 10: Reduction of residual enzyme activity at non-tumour sites

It is desirable to inactivate the enzymatic portion of the enzyme-antibody conjugate at non-tumour sites. but not at the tumour. One method of achieving this effect is to administer to the patient being treated using the compounds of the invention antibodies raised against the enzyme portion which have been conjugated with galactose residues.

A monoclonal antibody directed at CPA inactivates the enzyme. To prevent the antibody inactivating the enzymes at tumour sites additional galactose residues are conjugated to it so that it can still inactivate enzyme in plasma when it is given by intravenous route but the inactivating antibody is rapidly removed from the plasma and galactose receptors on hepatocytes.

The galactosylated anti-CPA monoclonal antibody is given to eliminate enzymic activity in plasma and then to give an amount of the non-galactosylated anti-CPA monoclonal antibody to inactivate residual enzyme activity in other non-tissues.

The monoclonal antibody is galactosylated using the following protocol: A stock solution of the activated derivative was made up as follows: Cyanomethyl 2,3,4,6-tetra-O-acetyl-l-thio-β-D-galactopyranoside (Sigma C-4141) [400 mg] in anhydrous methanol (10 ml) was treated with 5.4 mg of sodium methoxide in 1 ml of anhydrous methanol at room temperature for 48 hours. The mixture was kept in a 25 ml Quickfit conical flask fitted with a slightly greased stopper.

A stock solution of monoclonal antibody (1.3 mg/ml) is prepared in 0.25 M sodium borate buffer, pH 8.5. Aliquots of the required amount of activated galactosyl derivative (80, 40, 20, 10 μl) are dispensed into 3 ml glass ampoules and evaporated to a glassy residue in a stream of nitrogen. A solution of the antibody (200 μg) is added mixed until the residue is dissolved. After 2 hours at room temperature, the solution is dialysed against three changes of PBS.

The preparations are scaled up by taking multiples of the volumes mentioned above.

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6. Kuefner *et al* (1989) *Biochemistry* **28**, 2288-2297.

7. O'Sullivan *et al* (1979) *Anal. Biochem.* **100**, 100-108.

8. H. Zola (CRC Press, 1988) "Monoclonal Antibodies: A manual of techniques".

9. Melton *et al* (1987) *Biochem. Pharm.* **36(1)**, 105-112.

10. Melton *et al* (1987) *Biochem. Pharm.* **36(1)**, 113-121.

11. Köhler & Milstein (1975) *Nature* **256**, 495.

CLAIMS

1. A compound comprising a portion capable of at least partly restraining the compound from leaving the vascular compartment of a host when said compound is administered to the vascular compartment, and an inactivating portion capable of converting a cytotoxic agent into a less toxic substance.

2. A compound according to Claim 1 wherein the portion capable of restraining the compound from leaving the vascular compartment of a host is a macromolecular portion.

3. A compound according to Claim 2 wherein the macromolecular portion is a macroglobulin, liposome, dextran or other high molecular weight polymer.

4. A compound according to Claim 1 wherein the portion capable of restraining the compound from leaving the vascular compartment of a host is a red blood corpuscle.

5. A compound according to any one of the preceding claims wherein the inactivating portion comprises an enzymatically active portion.

6. A compound according to Claim 5 wherein the inactivating portion comprises at least the catalytic portion of carboxypeptidase G2 which is capable of degrading the cytotoxic agents methotrexate and aminopterin to less toxic substances.

7. A compound according to Claim 5 wherein the inactivating portion comprises at least the catalytic portion of glutathione-S-transferase

which is capable of inactivating cytotoxic alkylating agents to less toxic substances.

8. A compound according to any one of Claims 1 to 4 wherein the inactivating portion comprises an antibody or part thereof which binds to the cytotoxic agent.

9. A compound according to any one of Claims 1 to 4 wherein the inactivating portion comprises a thiol which binds to the cytotoxic agent.

10. A compound according to Claim 9 wherein the thiol is glutathione or an analogue thereof.

11. A method of at least partially destroying a cytotoxic agent in a vascular compartment of a host comprising administering to the host a compound according to any one of Claims 1 to 10.

12. A three component kit of parts comprising a first component comprising a target cell-specific portion and an enzymatically active portion capable of converting a cytotoxic pro-drug into a cytotoxic drug; a second component that is a cytotoxic pro-drug convertible by said enzymatically active portion to the cytotoxic drug; and a third component comprising a portion capable of at least partly restraining the component from leaving the vascular compartment of a host when said compound is administered to the vascular compartment. and an inactivating portion capable of converting the cytotoxic drug into a less toxic substance.

13. A kit of parts according to Claim 12 further comprising a means of

removing or inactivating the enzymatically active portion of said first component at non-target cell sites.

14. A kit of parts according to either one of Claims 12 and 13 wherein the target cell-specific portion comprises an antibody or part thereof.

15. A kit of parts according to any one of Claims 12 to 14 wherein the target cell-specific portion recognises and selectively binds to a tumour cell.

16. A kit of parts according to any one of Claims 12 to 15 wherein the enzymatically active portion of the first component comprises at least the catalytic portion of carboxypeptidase A.

17. A kit of parts according to Claim 16 wherein the drug is methotrexate or aminopterin or a quinazoline antifolate.

18. A kit of parts according to Claim 17 wherein the inactivating portion of the third component is at least the catalytic portion of carboxypeptidase G2.

19. A kit of parts according to any one of Claims 12 to 15 wherein the drug is an alkylating agent.

20. A kit of parts according to Claim 19 wherein the inactivating portion is a thiol.

21. A kit of parts according to Claim 19 wherein the inactivating portion is at least the catalytic portion of glutathione-S-transferase.

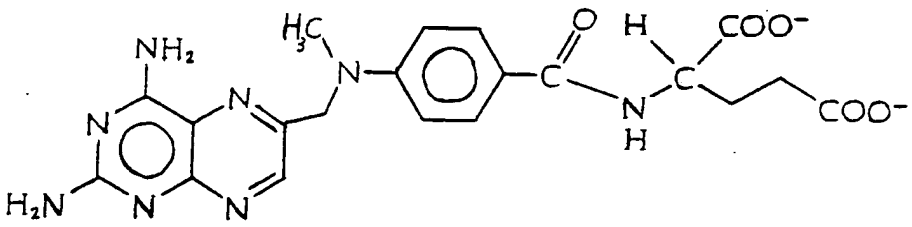
22. A kit of parts according to any one of Claims 12 to 18 wherein the restraining portion of the third component is a macroglobulin, liposome, high molecular weight polymer or red blood corpuscle.

23. A kit of parts according to any one of Claims 12 to 22 for use in a method of destroying target cells in a host involving administration to the host of the various components.

24. A method of destroying target cells in a host, the method comprising administering to the host (i) a first component as defined in any one of Claims 12 to 15, (ii) a second component as defined in any one of Claims 12, 13, 17 and 19 and (iii) a third component as defined in any one of Claims 12, 13, 18 and 20 to 22.

25. A method according to Claim 24 wherein each component is administered by an intravenous route.

METHOTREXATE



FOLINIC ACID

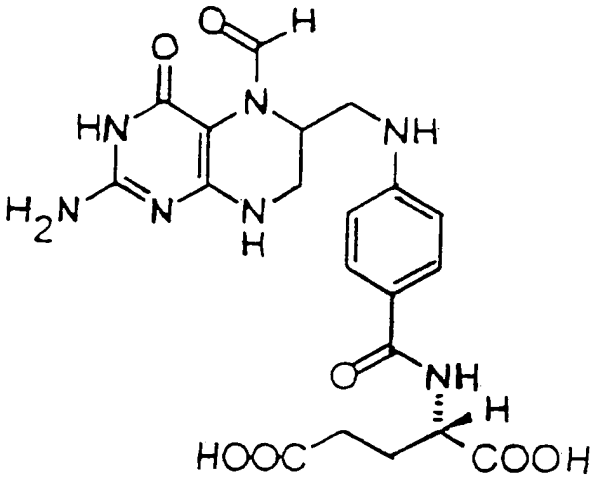
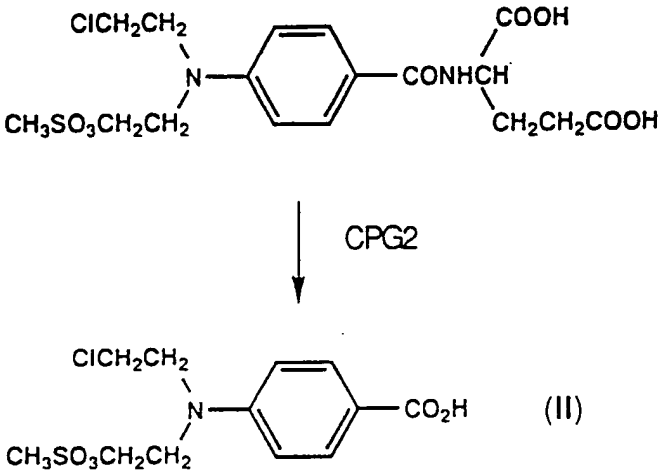


FIGURE 1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COOH
CONHCH
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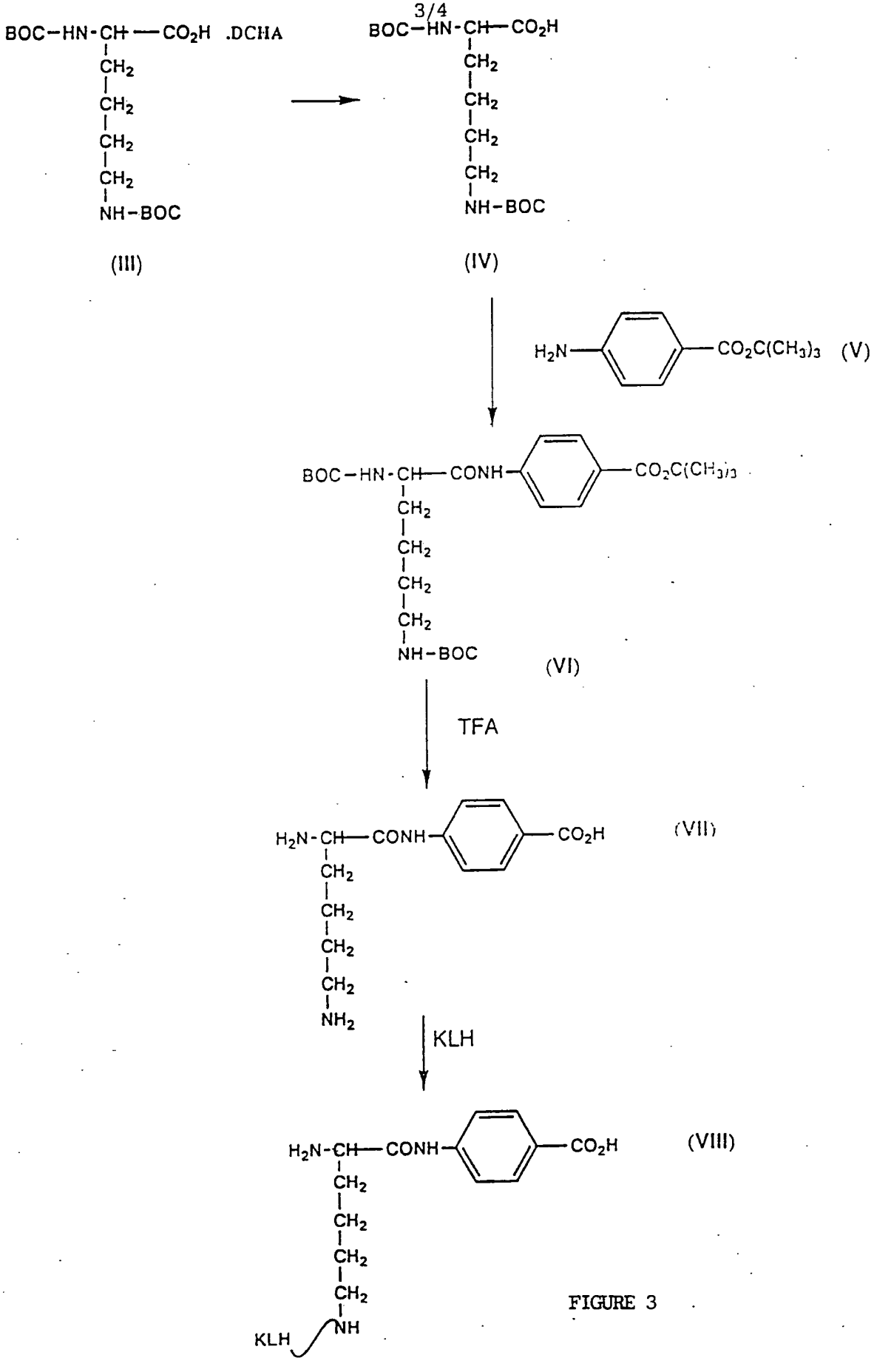
CO2H

(I)

(II)

FIGURE 2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CH2
CH2
CH2
NH-BOC
(III)
BOC-HN-CH-CO2H
CH2
CH2
CH2
CH2
NH-BOC
(IV)
H2N
CO2C(CH3)3
(V)
BOC-HN-CH-CONH
CO2C(CH3)3
CH2
CH2
CH2
CH2
NH-BOC
(VI)
TFA
H2N-CH-CONH
CO2H
(VII)
CH2
CH2
CH2
CH2
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CH2
KLH
NH

FIGURE 3

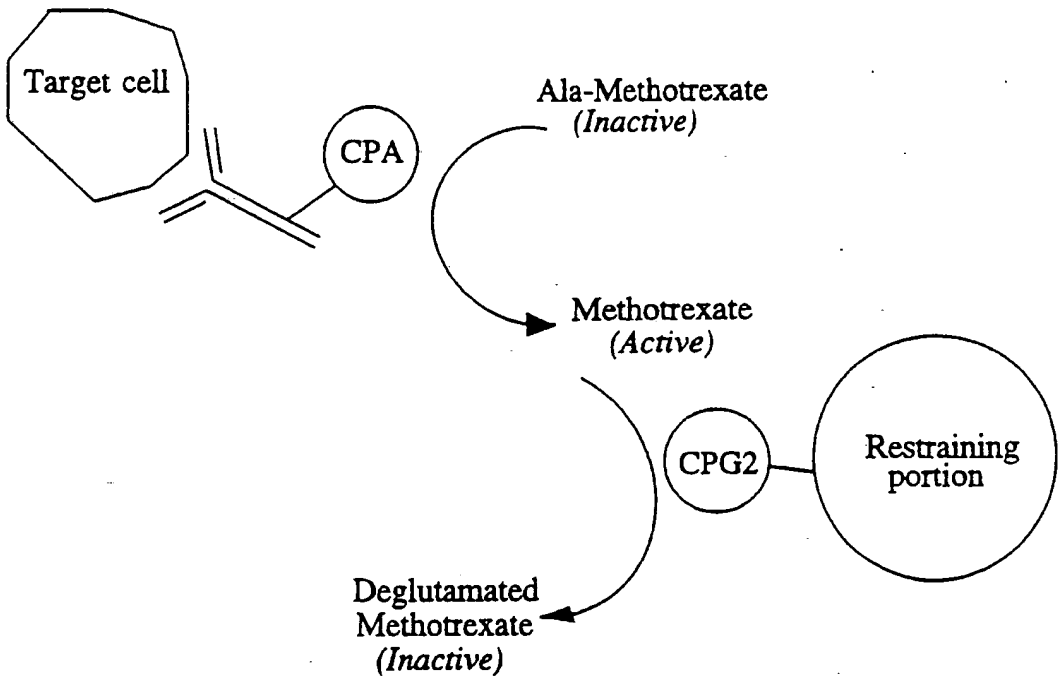
Target cell
CPA
Ala-Methotrexate
(Inactive)
Methotrexate
(Active)
CPG2
Restraining
portion
Deglutamated
Methotrexate
(Inactive)

FIGURE 4

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 93/00040

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 A61K47/48

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	A61K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
Y,X	WO,A,8 910 140 (CANCER RESEARCH CAMPAIGN TECHNOLOGY LTD) 2 November 1989 cited in the application see page 15, line 6 - line 14; claims; examples 3,5 ---	1-25
X,Y	PROC. NAT.ACAD.SCI. USA vol. 80, May 1983, pages 3078 - 3080 G. W. YU ET AL. 'MODEL FOR SPECIFIC RESCUE OF NORMAL HEPATOCYTES DURING METHTREXATE TREATMENT OF HEPATIC MALIGNANCY.' ---	1-25
X	WO,A,9 010 460 (MATSUMURA) 20 September 1990 see page 3, paragraph 3; claims 1-7 ---	1-25

-/--

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
13 MAY 1993	01. 07. 93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	BERTE M.J.

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	EP,A,0 308 208 (CENTER FOR MOLECULAR MEDICINE AND IMMUNOLOGY) 22 March 1989 see page 3, paragraph 2; claims	1-25
A	WO,A,8 807 378 (CANCER RESEARCH CAMPAIGN TECHNOLOGY LTD.) 6 October 1988 cited in the application see claims	1-25
X,P	EP,A,0 506 124 (TANOX BIOSYSTEMS) 30 September 1992 see claims	1-3
X	WO,A,9 109 134 (TAKEDA CHEMICAL INDUSTRIES LTD.) 27 June 1991 see claims	1
X	CHEMICAL ABSTRACTS, vol. 109, no. 17 Columbus, Ohio, US; abstract no. 142106q, see abstract & ANTIBODY, IMMUNOCONJUGATES, RADIOPHARM. vol. 1, no. 2, 1988, pages 169 - 180 J. BARBET ET AL. 'SPECIFIC TOXICITY TO ACTIVATED T AND B LYMPHOCYTES OF A RICIN A IMMUNOTOXIN DIRECTED AGAINST THE CLASS I MHC ANTIGEN, H-2 K.'	1-3,8,11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB93/00040

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Arucle 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1-25
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

In view of the great number of compounds for wich protection is being sought as recited in claims 1,12 and subsequent claims, the search has been restricted for economical reasons to components of this claim and subsequent claims (portion capable of at least...restraining the compound..,inactivati

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

GB 9300040
SA 68720

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 13/05/93

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-8910140	02-11-89	EP-A-	0414741	06-03-91
		JP-T-	3503898	29-08-91
WO-A-9010460	20-09-90	AU-A-	5349290	09-10-90
		EP-A-	0464135	08-01-92
EP-A-0308208	22-03-89	US-A-	4859449	22-08-89
		AU-A-	2223988	16-03-89
		JP-A-	1221400	04-09-89
WO-A-8807378	06-10-88	EP-A-	0408546	23-01-91
EP-A-0506124	30-09-92	AU-A-	1299292	15-10-92
WO-A-9109134	27-06-91	EP-A-	0505566	30-09-92